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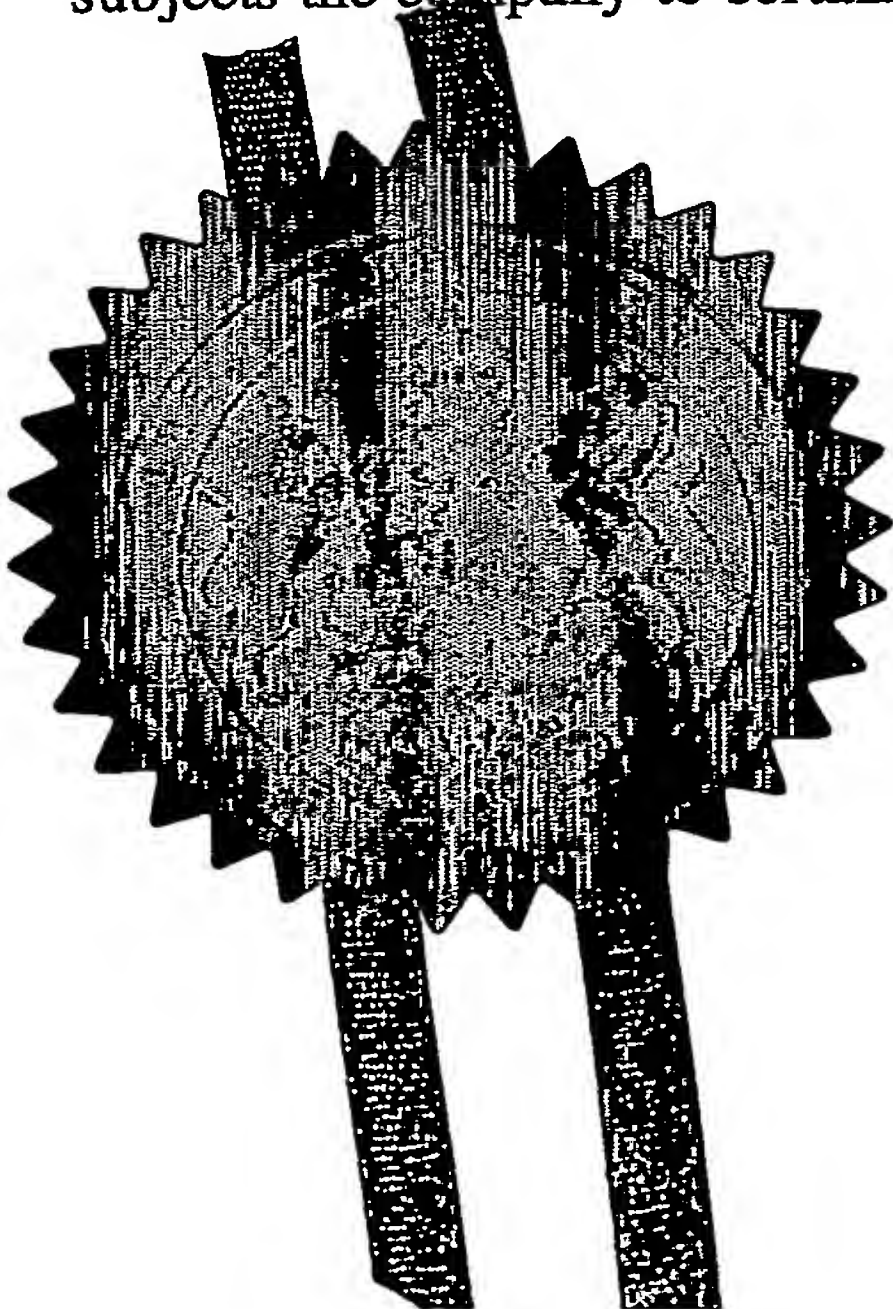
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Isis Innovation Limited,  
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0327742.3

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4. Title of the invention Novel uses of known drugs

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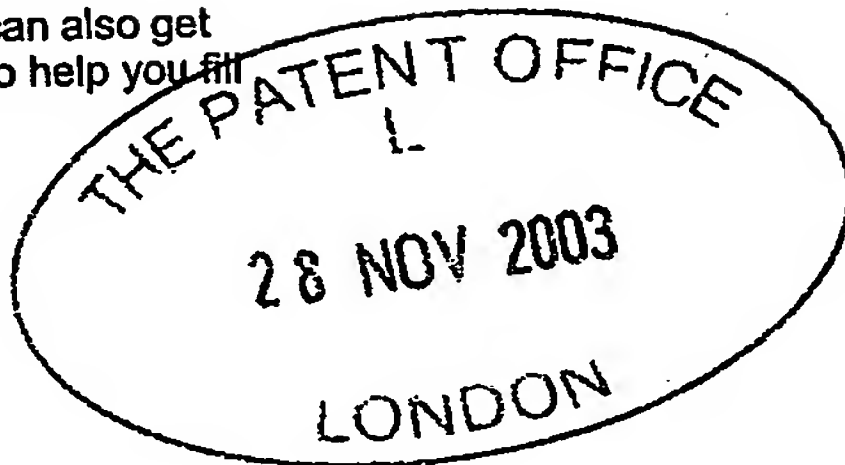
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Continuation sheets of this form	0
Description	14
Claim(s)	2 <i>DL</i>
Abstract	1
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Priority documents	
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Statement of inventorship and right to grant of a patent (Patents Form 7/77)	
Request for preliminary examination and search (Patents Form 9/77)	1 <i>✓</i>
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11. I/We request the grant of a patent on the basis of this application.

Signature(s) *Markus J. L. M.*

Date: 28 November 2003

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Patent Chemical Formalities  
020 7400 3000

# NOVEL USES OF KNOWN DRUGS

The present invention relates to the use of statins in medicine.

The bisphosphonates are useful in various clinical applications worldwide (1), particularly in the treatment of musculoskeletal disorders, and they are now amongst the most commonly used agents in clinical practice. They have proven to be particularly useful in the management of osteoporosis, but also in other disorders of the bone and joint, such as Paget's disease, skeletal metastases, fibrous dysplasia, Charcot's arthropathy, sympathetic dystrophy, pachydermic periostosis and aseptic osteomyelitis.

Bisphosphonates (BPs) are commonly used for the treatment of Paget's disease, multiple myeloma, osteoporosis and hypercalcaemia (reviewed in (1)). The clinical success of the BPs etidronate and clodronate, in the 1970s and 1980s, led to trials of BPs with different alkyl chains. In particular, bisphosphonates containing an amino group in their alkyl chain, such as pamidronate and alendronate, were found to be 10- to 100-fold more potent at inhibiting bone resorption than the early non-amino bisphosphonates (15). Risedronate and zoledronate contain a nitrogen atom within a heterocyclic ring (16) and have been shown to be up to 10,000-fold more potent than etidronate, in experimental systems (17).

Mechanistic studies indicate that the bisphosphonates can, broadly speaking, be classified into two groups, based on their mode of action: those that resemble pyrophosphate, including etidronate, clodronate, and tiludronate, and which can be incorporated into cytotoxic ATP analogues; and the more potent nitrogen-containing bisphosphonates that interfere with other metabolic pathways, such as the mevalonate pathway (reviewed in (1)).

Inhibition of the mevalonate pathway by nBPs leads to an accumulation of intermediates, including isopentenyl pyrophosphate (IPP), in this pathway (18). IPP is a potent activator of human peripheral blood  $\gamma\delta$ T cells (19) and nBPs have also been described as activating these cells (18, 20-23). The acute phase response has not been observed with the non-aminobisphosphonates, etidronate, clodronate or tiludronate, and appears to be a specific feature of the nBPs (1).

The dominant subset of human  $\gamma\delta$  T cells in peripheral blood bear T cell receptors (TCRs) encoded by the V $\gamma$ 9 and V $\delta$ 2 genes. These cells are known to activate in response to alkylphosphate, alkylamine and some bisphosphonate antigens. This recognition is a characteristic of cells bearing a V $\gamma$ 9 chain that uses the JP joining region (24). V $\gamma$ 9JP-expressing cells are highly enriched in the peripheral blood, compared to percentages in thymocytes or cord blood (24, 25), which is suggestive of antigen-induced expansion.

Bisphosphonates, and in particular the aminobisphosphonates (nitrogen-containing bisphosphonates, or nBPs), are known to have a number of side effects (reviewed in (1)) including a rise in body temperature and accompanying flu-like symptoms which resemble a typical acute phase response (2, 3), and was first described over 15 years ago. The mechanism for this response has been partially elucidated and appears to be associated with the release of tumour necrosis factor alpha (TNF $\alpha$ ) and IL6 (4-6), although the effector cells that release these cytokines and the mechanism of action remain unknown.

The cellular mechanism for the action of nBPs leading to production of TNF $\alpha$  and IL6 has remained unclear, although monocytes and/or macrophages have been mooted as likely candidates for releasing these pro-inflammatory agents (5). In addition, there has been considerable concern over gastrointestinal disturbances associated with oral administration of the nBP alendronate (28). Similar problems had earlier led to the discontinuation of oral pamidronate for osteoporosis (29), although administration of these compounds by intravenous or intramuscular injection is increasing in popularity worldwide, as these routes avoid gastrointestinal disturbances. It would clearly be advantageous to find a way to be able to administer nBPs orally, once again, without the above-identified problems.

Bukowski *et al.* (31) demonstrated that a single V $\gamma$ 9V $\delta$ 2 T cell clone could recognise two types of ligand, alkylamines and IPP, and suggested that, given the small sizes of these antigens, a single  $\gamma\delta$  TCR might possess two different binding sites. The similarity between the recognised ligands is their alkyl chains. While it is known that phosphate antigens require their phosphate and nBPs and alkylamines require their amino groups for their antigenicity (1, 19, 20) which, it has been assumed, forms a component of the TCR-docking structure. The finding that both antigenic

alkylphosphate and alkylamine ligands require similar alkyl chains for recognition strengthened this assumption, and lead to attempts at molecular prediction (27).

The most potent nBP antigen to date, risedronate, which can stimulate  $\gamma\delta$  T cell expansion from human PBMC at concentrations as low as 10 nM (21) was not included in this predictive exercise (27). Risedronate has an aromatic ring and does not fit with the pattern recognition hypothesis championed by Gossman and Oldfield (27).

Surprisingly, it has now been established that the nBPs are not acting as an antigen at all, which helps to explain why risedronate does not fit the pattern recognition hypothesis, but are acting *via* their effect on the mevalonate pathway. Thus, rather than acting directly on monocytes and/or macrophages, it is their ability to raise levels of intermediary metabolites found in the mevalonate pathway that stimulates the acute phase effect and, to this extent, it has also been found that the effect is mediated by the  $\gamma\delta$  T cells. This effect can be ameliorated or blocked by blocking the mevalonate pathway at a stage before the activating intermediates are produced.

Thus, in a first aspect, the present invention provides a pharmaceutical formulation comprising an early-stage mevalonate pathway blocker and a bisphosphonate.

In an alternative aspect, the present invention provides the use of an early-stage mevalonate pathway blocker and a bisphosphonate in the manufacture of a medicament for the treatment or prophylaxis of a condition treatable by the bisphosphonate.

In a further aspect, there is provided the use of an early-stage mevalonate pathway blocker in the manufacture of a medicament for the treatment or prophylaxis of side effects associated with a bisphosphonate.

There is also provided a kit comprising a first medicament comprising an early-stage mevalonate pathway blocker and a second medicament comprising bisphosphonate, preferably together with an indication that the first medicament should be taken no later than the second.

The gastrointestinal tract is the main reservoir of  $\gamma\delta$  T cells in the body, as it is the main portal of entry for bacterial pathogens. Thus, oral administration of the medicaments of the present invention serves to provide protection against the effect of

orally administered nBPs, although it will be appreciated that circulatory  $\gamma\delta$  T cells are generally considered to be those that provide the acute phase response. Indeed, the acute phase response is still observed with intravenous administration of nBPs.

It will be appreciated that any suitable substance suitable for therapeutic administration may be used as an early-stage blocker of the mevalonate pathway. In this respect, "early-stage" indicates that the pathway should be blocked at a stage before any significant amount of  $\gamma\delta$  activating intermediates is produced. In particular, it has been established that IPP activates  $\gamma\delta$  T cells, so that it is preferred to block the pathway at a point before the production of IPP. It is more preferred to block at an earlier stage, in order to minimise the possibility of a build up of an earlier intermediate forcing the equilibrium toward IPP.

Suitable substances for use in the present to block the mevalonate pathway at an early stage are those substances identified as the statins. The statins are inhibitors of 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMG-CoA reductase), an enzyme which catalyses the conversion of HMG-CoA to mevalonate, which is a required building block for cholesterol biosynthesis. The statins do not all share a similar structure. For example, simvastatin and lovastatin vary by only one methyl, while there is little similarity with atorvastatin or fluvastatin. Likewise, mevinolin shares some similarity with lovastatin, but has a substantially different side-chain. Nevertheless, the skilled person is familiar with the statins, and is readily able to select a statin suitable for administration with the bisphosphonate. Any one of the statins may be used alone, or together with any one or more other statins.

Where statins are referred to herein, it will be appreciated that other early-stage mevalonate pathway blockers are also envisaged, unless otherwise apparent or indicated.

In general, the bisphosphonates whose effect it is desired to counteract will be the nBPs, and this group is particularly preferred, especially those recognised for their clinical utility. Preferred nBPs include pamidronate, dimethyl pamidronate, and alendronate, which contain a basic primary nitrogen atom in an alkyl chain, and ibandronate, risedronate and zoledronate, which contain a tertiary nitrogen. Any one of the nBPs may be used alone, or together with any one or more other nBPs.

Although the statins may be administered together with the nBPs, it will be appreciated that greater efficiency at blocking the pathway will be achieved by earlier administration. In particular, as the pathway is generally constitutive, blocking it at the beginning will not have an effect downstream for some time, so that a statin co-administered with an nBP will prevent further HMG-CoA entering the pathway, but that which is already on the pathway can still be blocked by the nBP, leading to an accumulation of IPP. However, the overall quantities will be less, and it may often be sufficient to co-administer the statin and nBP.

However, it is preferred to administer the statin from several hours before the nBP to several days before. Where the product is a kit, for example, it is preferred to indicate this on the pack, and to provide extra medicament comprising statin, for the purpose.

It will also be appreciated that, after the initial statin application or course, it will generally be acceptable to co-administer the statin with the nBP for the remainder of the treatment.

It is a particular advantage of the present invention that it is now possible to administer nBPs orally with substantially reduced likelihood of acute phase side effects. Thus, oral preparations are preferred, and include such formulations as tablets, capsules, elixirs and syrups, and may contain suitable excipients and flavourings. Tablets may be formulated simply with a carrier or may be enterically coated, for example.

Suitable quantities of statins and nBPs for use in the invention will be readily discerned by those skilled in the art, and will generally correspond to those known to be effective for the, or other, indications of the compounds. It is generally desirable to use the same or higher concentrations of the statins, at least to start, and preferably for the duration, than are used to control levels of cholesterol in a patient.

As verification of the present invention, a clonal population of V $\gamma$ 9JPV $\delta$ 2 T cells was shown to activate after exposure to the nBPs, pamidronate and risedronate, but not the nonaminoBPs, etidronate and clodronate, by INF $\gamma$  ELISpot (Figure 1A). V $\gamma$ 9JPV $\delta$ 2 T cells also made large amounts of TNF $\alpha$  after exposure to nBPs (Figure 1C). Production of TNF $\alpha$  was both copious and rapid (Figure 1C). A V $\gamma$ 9JPV $\delta$ 1-

expressing clone was unable to recognise these ligands, indicating that the  $\delta 2$  chain also plays an essential role in the response of  $V\gamma 9JPV\delta 2$  T cells to nBPs.

Pamidronate and risedronate were also able to induce large amounts of  $TNF\alpha$  from direct *ex vivo* human peripheral blood mononuclear cells (PBMC) (Figure 1B). Production of this cytokine was rapid (Figure 1B). Magnetic depletion of  $\gamma\delta$  T cells removed the ability of PBMC to produce  $TNF\alpha$  in response to pamidronate (Figure 2A) and risedronate (Figure 2B). Intracellular cytokine staining (ICS) showed that exposure of PBMC to nBPs activated the peripheral blood lymphocyte population bearing a  $V\gamma 9$  TCR and not other T cells or B cells (Figure 2C).  $V\gamma 9$ -expressing cells were shown to make both IL6 and  $TNF\alpha$  in response to pamidronate and risedronate by ICS (partially shown in Figure 2D).

A previous study (26) found that 4 in 10 patients given 60 or 90 mg infusions of pamidronate had an acute phase reaction. All of these patients showed a substantial increase in the number of circulating  $\gamma\delta$  T cells when measured 1 and 3 weeks post-infusion (26). In one patient  $\gamma\delta$  T cells expanded from 4.6% to 70% of  $CD3+$  cells post-infusion.

In order to demonstrate that nBPs exert their effect on the activation of  $V\gamma 9JPV\delta 2$  T cells *via* the mevalonate pathway, and not to act as direct ligands for the  $\gamma\delta$  TCR as had been thought in previous studies (20-23, 27), inhibitors of HMG CoA were shown to be able to inhibit the nBP-induced activation of  $\gamma\delta$  T cells directly *ex vivo* (Figure 3).

Pravastatin, simvastatin and fluvastatin were all able to inhibit  $TNF\alpha$  production by fresh PBMC, as measured by ELISA (Figure 3A). Addition of IPP was able to rescue  $TNF\alpha$  production, ruling out the possible toxic effects of these statins at the concentrations used. These statins were also shown to inhibit the activation of  $V\gamma 9$  cells by ICS (Figure 3B). Once again, addition of IPP in addition to the statin was able to rescue activation and rule out toxic effects.

It has now been shown that nBPs induce rapid and copious production of the pro-inflammatory cytokines by human peripheral  $\gamma\delta$  T cells (Figures 1-3), with depletion of  $\gamma\delta$  T cells from PBMC abrogating this effect (Figure 2), and that statins,

which inhibit HMG CoA reductase, are able to inhibit nBP-induced activation of  $\gamma\delta$  T cells (Figure 3).

It is further noteworthy that statins have anti-inflammatory properties (7-14) and appear to promote a Th2 cytokine bias *in vivo* (30); the therapeutic relevance of these pharmacologic effects is becoming increasingly apparent. The mechanism of such effects is still under debate. Our work indicates that some of the anti-inflammatory effects of these compounds could potentially result from their ability to inhibit the activation of  $\gamma\delta$  T cells, a result that could shed light on the physiological relevance of these lymphocytes in immune regulation.

The present invention will now be illustrated by the following, non-limiting Example.

### EXAMPLE

In the following Example, the materials and methods were as follows:

#### *Drugs*

The following bisphosphonates and statins were used: disodium etidronate (Procter & Gamble Pharm), disodium clodronate (Roche), disodium pamidronate (Novartis), risedronate sodium (Procter & Gamble Pharm), simvastatin (Ranbaxy Laboratories Limited), pravastatin (Bristol-Myers Squibb Pharmaceuticals) and fluvastatin (Sandoz Pharmaceuticals).

#### *$\gamma\delta$ T cell culture*

$\gamma\delta$  T cells were isolated from human peripheral blood using a magnetic separation kit (TCR  $\gamma/\delta$  Microbead Kit and MS columns from Miltenyi Biotec). Once isolated, cells were resuspended in T cell medium (RPMI supplemented with 100U/ml penicillin, 100  $\mu$ g/ml streptomycin, 10% FCS, 10% T-STIM (BD Biosciences), 200U/ml Proleukin (Chiron)) containing  $2 \times 10^6$   $\gamma$ -irradiated PMBC/ml from three individuals and 2  $\mu$ g/ml PHA. Cells were maintained with T cell medium, and restimulated with mixed irradiated PBMC and PHA every three weeks. For cloning, the same mix was used to grow cells by limiting dilution in 96-well U-bottomed plates. Cells that grew were confirmed to be  $\gamma\delta$  T cells with V $\gamma$ 9 antibody.

### *Antibodies*

The following antibodies were used for flow cytometric analysis: FITC-conjugated mouse anti-human V $\delta$ 2 TCR mAb clone B6.1 (PharMingen), FITC-conjugated mouse anti-human V $\gamma$ 9 mAb clone 7A5 (Endogen), FITC-conjugated mouse anti-human V $\delta$ 1 mAb clone TS8.2 (Endogen), PE-conjugated mouse anti-human V $\gamma$ 9 mAb clone B3.1 (PharMingen), FITC-conjugated mouse anti-human pan  $\gamma\delta$  mAb clone 5A6.E9 (Endogen), PerCP-conjugated mouse anti-human CD3 mAb clone SK7 (BD Biosciences), allophycocyanin (APC)-conjugated anti-human IL2 clone MQ1-17H12 (Caltag), APC-conjugated anti-human TNF $\alpha$  clone mAb11 (PharMingen), APC-conjugated anti-human IFN $\gamma$  clone B27 (PharMingen) and PE-conjugated anti-human IL6 clone AS12 (BD Biosciences).

### *$\gamma$ -IFN ELISpot and TNF $\alpha$ ELISA*

$\gamma\delta$  T cells were washed in RPMI and incubated overnight in R10 at 37°C. 96-well nitrocellulose plates (Millipore) were incubated overnight at 4°C with 15  $\mu$ g/ml antihuman-IFN- $\gamma$  primary antibody (clone 1-D1K; Mabtech, Sweden). The plates were then washed twice with RPMI and blocked with R10 for 3 hours at 37°C. R10 was decanted by inversion and assays applied to each well before incubation at 37°C as detailed below. Assays were terminated by washing once in water, followed by 5 washes in PBS. Secondary antibody (anti-human-IFN- $\gamma$ -Biotin antibody clone 7-B6-1; Mabtech) was applied at 1  $\mu$ g/ml and the plate incubated for 100 minutes at room temperature (RT). The plate was washed 6 times with PBS before application of Streptavidin-ALP (1:1000 in PBS; Mabtech) for 40 minutes at RT. After 6 further washes in PBS, spots were revealed by incubation for 15 minutes at RT with developing buffer (Bio-Rad AP conjugate substrate kit) and counted mechanically using an ELISpot Reader System ELR02 (Autoimmun Diagnostika; Strassberg). A TNF $\alpha$  ELISA kit (Peprotech) was used in accordance with the manufacturers instructions.

### *Intracellular cytokine staining*

10<sup>6</sup> fresh PBMC were incubated in FACS tubes with brefeldin A (10  $\mu$ g/ml R10) for 5 hours after pulsing with relevant antigens for 1 hour. The cells were then washed, permeabilised in 20% FACSlyse (BD Biosciences), washed twice in ice cold PBS/0.1% BSA and stained on ice with pre-titred APC-conjugated anti-IFN $\gamma$ , anti-

TNF $\alpha$  and anti-IL-2 and PE-conjugated anti-V $\gamma$ 9 mAbs for 20 minutes. For single cytokine analysis (Figure 2D), cells were stained with PE-conjugated anti-IL6, APC-conjugated anti-TNF $\alpha$  and FITC-conjugated anti-V $\gamma$ 9 antibodies. In each case, cells were then washed, resuspended in PBS & analysed immediately.

#### *$\gamma\delta$ T cell depletion of PBMC*

$10^8$  human PBMC were incubated with 10  $\mu$ g/ml of anti-human pan- $\gamma\delta$  antibody for 30 minutes on ice. Cells were washed once in 20X labelling volume with PBS/0.1%BSA and resuspended at  $4 \times 10^7$  cells/ml in PBS/0.1%BSA.  $2 \times 10^7$  anti-mouse IgG1 Dynabeads (Dyna) washed in PBS/0.1%BSA were added and the cells incubated for 1hr at 4°C with gentle rotation. The tube was then placed in the magnetic particle concentrator (Dyna) and left to separate for 30 minutes. The supernatant was transferred to a fresh tube. The success of depletion was determined by FACS analysis with the anti-CD3 and pan- $\gamma\delta$  antibodies detailed above, and was >95% efficient in all cases shown.

The results are shown in the accompanying Figures.

Figure 1 shows that nBPs induce rapid and copious production of TNF $\alpha$  by peripheral blood  $\gamma\delta$  T cells.

In Figure 1 (A) nBPs, but not non-aminoBPs, are shown to activate a V $\gamma$ 9JPV $\delta$ 2 cell clone Bob in IFN $\gamma$  ELISpot. ELISpots were performed with 1,000 V $\gamma$ 9JPV $\delta$ 2 T cells and 25,000 spinner HeLa cells as antigen presenting cells per well and incubated for 6 hours prior to development. Standard error from the mean of two replicate assays is shown although, in all cases, these errors are smaller than the plot symbol.

In Figure 1 (B), nBPs are shown to stimulate TNF $\alpha$  production from direct *ex vivo* human PBMC.  $10^6$  fresh human PBMC were incubated for 12 hours in 75x5mm FACS tubes at 37°C and 5% CO $_2$  in 1 ml of R10 (RPMI, 10% FCS, 100 u/ml penicillin, 100  $\mu$ g/ml streptomycin), 1 ml of R10 + 10  $\mu$ M risedronate or 1 ml of R10 + 100  $\mu$ M pamidronate. 60  $\mu$ l aliquots were removed at the specified time and added to TNF $\alpha$  ELISA assays.

In Figure 1 (C), activation of V $\gamma$ 9JPV $\delta$ 2 T cell clones by risedronate is shown to be extremely rapid. The standard deviation from the mean of two replicate TNF $\alpha$  assays is shown for three separate clones expressing a V $\gamma$ 9JPV $\delta$ 2 TCR. Clone P is CD8 $\alpha$ +, Clones M and Bob are CD8-. Clone P appears to make more TNF $\alpha$  and to produce it earlier.  $10^6$  T cells were activated by 10  $\mu$ M risedronate in 1 ml of media in 75x5mm FACS tube at 37°C and 5% CO $_2$ . 60  $\mu$ l aliquots were removed at the specified time and added to TNF $\alpha$  ELISA assays.

Figure 2 shows how the TNF $\alpha$  and IL6 produced by PBMC in response to nBPs is derived from  $\gamma\delta$ T cells.

Magnetic depletion of  $\gamma\delta$ T cells from human PBMC removes their ability to manufacture TNF $\alpha$  in response to 100  $\mu$ M pamidronate (A) and 10  $\mu$ M risedronate (B).  $5 \times 10^6$  PBMC from a healthy donor +/- magnetic depletion of  $\gamma\delta$  T cells were suspended in 1 ml of R10 +/- antigen in 75x5mm FACS tubes at 37°C and 5% CO $_2$  for the times shown. 60  $\mu$ l of cell supernatant was removed and applied to TNF $\alpha$  ELISA plates in duplicate and developed according to the manufacturers instructions. Standard deviation from the mean of the two replicate ELISAs is shown although, in most cases, this error is smaller than the plot symbol. In (C), exposure to nBPs activates PBMC that express a V $\gamma$ 9 receptor and not other T cells or B cells.  $10^6$  fresh human PBMC were exposed to R10 (top panel) or R10 + 10  $\mu$ M risedronate (bottom panel) for 6 hours in an intracellular cytokine staining (ICS) assay. Plots show all the cells in the lymphocyte gate stained for PE-V $\gamma$ 9 and APC-cytokines (TNF $\alpha$ , IL2, and IFN $\gamma$ ). Exposure to risedronate induces cytokine production only in lymphocytes that express a V $\gamma$ 9 TCR. The percentage of total lymphocytes in the V $\gamma$ 9 $^+$  cytokine $^+$  gate shown is indicated in the upper right of each panel. Almost 10% of the lymphocytes expressing a V $\gamma$ 9 receptor are activated by exposure to nBPs. Similar results were observed with 100  $\mu$ M pamidronate (data not shown). It is noticeable that exposure to risedronate lowers the expression of the V $\gamma$ 9 TCR. In (D), intracellular cytokine staining (ICS) shows that nBPs induce direct *ex vivo* V $\gamma$ 9-expressing T cells to make TNF $\alpha$  and IL6. Plots are gated to show only V $\gamma$ 9-expressing lymphocytes. The left hand panels show IL6 production induced by 100  $\mu$ M pamidronate and the right hand panels show TNF $\alpha$  production induced by 10  $\mu$ M risedronate. The percentage of cytokine positive cells (fluorescence intensity >20) is shown in the upper right corner of each panel.

Figure 3 shows that statins inhibit nBP-induced TNF $\alpha$  production by human PBMC.

Figure 3 (A) shows the results of pre-treatment of human PBMC, where 1  $\mu$ M pravastatin, 100 nM simvastatin or 100 nM fluvastatin, for 2 hours, inhibits their ability to manufacture TNF $\alpha$  in response to 10  $\mu$ M risedronate. Addition of IPP restores TNF $\alpha$  production and provides a control against any possible toxic effects of these statins.  $5 \times 10^5$  cells/well in 200  $\mu$ l were incubated with nBP for 12 hours at 37°C and 5% CO<sub>2</sub> in 96 well U-bottomed tissue culture plates. 60  $\mu$ l of supernatant was applied to a TNF $\alpha$  ELISA plate (Preprotec). Assays were performed in duplicate. Bars show the standard deviation from the mean of two replicate assays.

In Figure 1 (B), it is shown that pre-treatment of PBMC with statins inhibits nBP-induced activation of V $\gamma$ 9-expressing T cells, by ICS. Results are shown for three separate experiments. The left panels show results with 1 mM pravastatin; results with 100 nM simvastatin and 100 nM fluvastatin are shown in the middle and right panels respectively. In each case, the experiments show results of 6 hours incubation in R10 only on the top row, R10 + 10  $\mu$ M risedronate in row 2, R10 + 10  $\mu$ M IPP in row 3, R10 + 10  $\mu$ M risedronate + statin in row 4 and R10 + 10  $\mu$ M risedronate + statin + 10  $\mu$ M IPP on the bottom row. Statins inhibit nBP-induced activation of V $\gamma$ 9-expressing T cells (compare rows 2&4). In each case, addition of IPP with the statin (bottom row) rescues activation and controls for any toxic effect of the statins. The percentage of lymphocytes judged to activate in each experiment is shown in the upper right of each panel.

## References

1. Russell, R. G., P. I. Croucher, and M. J. Rogers. 1999. Bisphosphonates: pharmacology, mechanisms of action and clinical uses. *Osteoporos Int* 9 Suppl 2:S66.
2. Adami, S., A. K. Bhalla, R. Dorizzi, F. Montesanti, S. Rosini, G. Salvagno, and V. Lo Cascio. 1987. The acute-phase response after bisphosphonate administration. *Calcif Tissue Int* 41:326.

3. Gallacher, S. J., S. H. Ralston, U. Patel, and I. T. Boyle. 1989. Side-effects of pamidronate. *Lancet* 2:42.
4. Schweitzer, D. H., M. Oostendorp-van de Ruit, G. Van der Pluijm, C. W. Lowik, and S. E. Papapoulos. 1995. Interleukin-6 and the acute phase response during treatment of patients with Paget's disease with the nitrogen-containing bisphosphonate dimethylaminohydroxypropylidene bisphosphonate. *J Bone Miner Res* 10:956.
5. Sauty, A., M. Pecherstorfer, I. Zimmer-Roth, P. Fioroni, L. Juillerat, M. Markert, H. Ludwig, P. Leuenberger, P. Burckhardt, and D. Thiebaud. 1996. Interleukin-6 and tumor necrosis factor alpha levels after bisphosphonates treatment in vitro and in patients with malignancy. *Bone* 18:133.
6. Thiebaud, D., A. Sauty, P. Burckhardt, P. Leuenberger, L. Sitzler, J. R. Green, A. Kandra, J. Zieschang, and P. Ibarra de Palacios. 1997. An in vitro and in vivo study of cytokines in the acute-phase response associated with bisphosphonates. *Calcif Tissue Int* 61:386.
7. Kobashigawa, J. A., S. Katznelson, H. Laks, J. A. Johnson, L. Yeatman, X. M. Wang, D. Chia, P. I. Terasaki, A. Sabad, G. A. Cogert, and et al. 1995. Effect of pravastatin on outcomes after cardiac transplantation. *N Engl J Med* 333:621.
8. Stuve, O., T. Prod'homme, A. Slavin, S. Youssef, S. Dunn, L. Steinman, and S. S. Zamvil. 2003. Statins and their potential targets in multiple sclerosis therapy. *Expert Opin Ther Targets* 7:613.
9. Stuve, O., S. Youssef, L. Steinman, and S. S. Zamvil. 2003. Statins as potential therapeutic agents in neuroinflammatory disorders. *Curr Opin Neurol* 16:393.
10. Marz, W., and W. Koenig. 2003. HMG-CoA reductase inhibition: anti-inflammatory effects beyond lipid lowering? *J Cardiovasc Risk* 10:169.
11. Crisby, M. 2003. Modulation of the inflammatory process by statins. *Drugs Today (Barc)* 39:137.
12. Leung, B. P., N. Sattar, A. Crilly, M. Prach, D. W. McCarey, H. Payne, R. Madhok, C. Campbell, J. A. Gracie, F. Y. Liew, and I. B. McInnes. 2003. A novel anti-inflammatory role for simvastatin in inflammatory arthritis. *J Immunol* 170:1524.

13. Blanco-Colio, L. M., J. Tunon, J. L. Martin-Ventura, and J. Egido. 2003. Antiinflammatory and immunomodulatory effects of statins. *Kidney Int* 63:12.
14. Weitz-Schmidt, G. 2002. Statins as anti-inflammatory agents. *Trends Pharmacol Sci* 23:482.
15. Shinoda, H., G. Adamek, R. Felix, H. Fleisch, R. Schenk, and P. Hagan. 1983. Structure-activity relationships of various bisphosphonates. *Calcif Tissue Int* 35:87.
16. Rogers, M. J., D. J. Watts, and R. G. Russell. 1997. Overview of bisphosphonates. *Cancer* 80:1652.
17. Fleisch, H. 1998. Bisphosphonates: mechanisms of action. *Endocr Rev* 19:80.
18. Gober, H. J., M. Kistowska, L. Angman, P. Jenö, L. Mori, and G. De Libero. 2003. Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. *J Exp Med* 197:163.
19. Tanaka, Y., C. T. Morita, E. Nieves, M. B. Brenner, and B. R. Bloom. 1995. Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature* 375:155.
20. Kunzmann, V., E. Bauer, J. Feurle, F. Weissinger, H. P. Tony, and M. Wilhelm. 2000. Stimulation of gammadelta T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. *Blood* 96:384.
21. Das, H., L. Wang, A. Kamath, and J. F. Bukowski. 2001. Vgamma2Vdelta2 Tcell receptor-mediated recognition of aminobisphosphonates. *Blood* 98:1616.
22. Kato, Y., Y. Tanaka, F. Miyagawa, S. Yamashita, and N. Minato. 2001. Targeting of tumor cells for human gammadelta T cells by nonpeptide antigens. *J Immunol* 167:5092.
23. Kato, Y., Y. Tanaka, H. Tanaka, S. Yamashita, and N. Minato. 2003. Requirement of species-specific interactions for the activation of human gamma delta T cells by pamidronate. *J Immunol* 170:3608.
24. Miyagawa, F., Y. Tanaka, S. Yamashita, B. Mikami, K. Danno, M. Uehara, and N. Minato. 2001. Essential contribution of germline-encoded lysine residues in

Jgamma1.2 segment to the recognition of nonpeptide antigens by human gammadelta T cells. *J Immunol* 167:6773.

25. Davodeau, F., M. A. Peyrat, M. M. Hallet, J. Gaschet, I. Houde, R. Vivien, H. Vie, and M. Bonneville. 1993. Close correlation between Daudi and mycobacterial antigen recognition by human gamma delta T cells and expression of V9JPC1 gamma/V2DJC delta-encoded T cell receptors. *J Immunol* 151:1214.

26. Kunzmann, V., E. Bauer, and M. Wilhelm. 1999. Gamma/delta T-cell stimulation by pamidronate. *N Engl J Med* 340:737.

27. Gossman, W., and E. Oldfield. 2002. Quantitative structure--activity relations for gammadelta T cell activation by phosphoantigens. *J Med Chem* 45:4868.

28. Graham, D. Y., H. M. Malaty, and R. Goodgame. 1997. Primary aminobisphosphonates: a new class of gastrotoxic drugs--comparison of alendronate and aspirin. *Am J Gastroenterol* 92:1322.

29. Lufkin, E. G., R. Argueta, M. D. Whitaker, A. L. Cameron, V. H. Wong, K. S. Egan, W. M. O'Fallon, and B. L. Riggs. 1994. Pamidronate: an unrecognized problem in gastrointestinal tolerability. *Osteoporos Int* 4:320.

30. Youssef, S., O. Stuve, J. C. Patarroyo, P. J. Ruiz, J. L. Radosevich, E. M. Hur, M. Bravo, D. J. Mitchell, R. A. Sobel, L. Steinman, and S. S. Zamvil. 2002. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* 420:78.

31. Bukowski, J. F., C. T. Morita, and M. B. Brenner. 1999. Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. *Immunity* 11:57.

**CLAIMS:**

1. A pharmaceutical formulation comprising an early-stage mevalonate pathway blocker and a bisphosphonate.
2. The use of an early-stage mevalonate pathway blocker and a bisphosphonate in the manufacture of a medicament for the treatment or prophylaxis of a condition treatable by the bisphosphonate.
3. The use of an early-stage mevalonate pathway blocker in the manufacture of a medicament for the treatment or prophylaxis of side effects associated with a bisphosphonate.
4. A kit comprising a first medicament comprising an early-stage mevalonate pathway blocker and a second medicament comprising bisphosphonate, preferably together with an indication that the first medicament should be taken no later than the second.
5. A formulation, use or kit, according to any preceding claim, wherein the early-stage mevalonate pathway blocker is a statin.
6. A formulation, use or kit, according to any preceding claim, wherein the statin is simvastatin, lovastatin, atorvastatin, fluvastatin, mevinolin, or a combination thereof.
7. A formulation, use or kit, according to any preceding claim, wherein the bisphosphonate is a nitrogen containing bisphosphonate.
8. A formulation, use or kit, according to any preceding claim, wherein the bisphosphonate is pamidronate, dimethyl pamidronate, alendronate, ibandronate, risedronate, zoledronate, or combination thereof.
9. A formulation, use or kit, according to any preceding claim, wherein the early-stage mevalonate pathway inhibitor is administered from several hours before the bisphosphonate to several days before.

10. The kit of claim 9, wherein there is an indication to take extra early-stage mevalonate pathway inhibitor before the bisphosphonate, and wherein extra medicament comprising early-stage mevalonate pathway inhibitor is provided.
11. A formulation, use or kit, according to any preceding claim, wherein the medicaments are provided in a form suitable for oral administration.

**ABSTRACT****NOVEL USES OF KNOWN DRUGS**

Statins are able to block the mevalonate pathway at an early stage, preventing build up of isopentenyl pyrophosphate by nitrogen containing bisphosphonates blocking the pathway further downstream, medicaments administered at an appropriate time thereby preventing the build up of IPP which has been found to activate  $\gamma\delta$  T cells and cause the acute phase response associated with the nitrogen containing bisphosphonates.

Figure 1

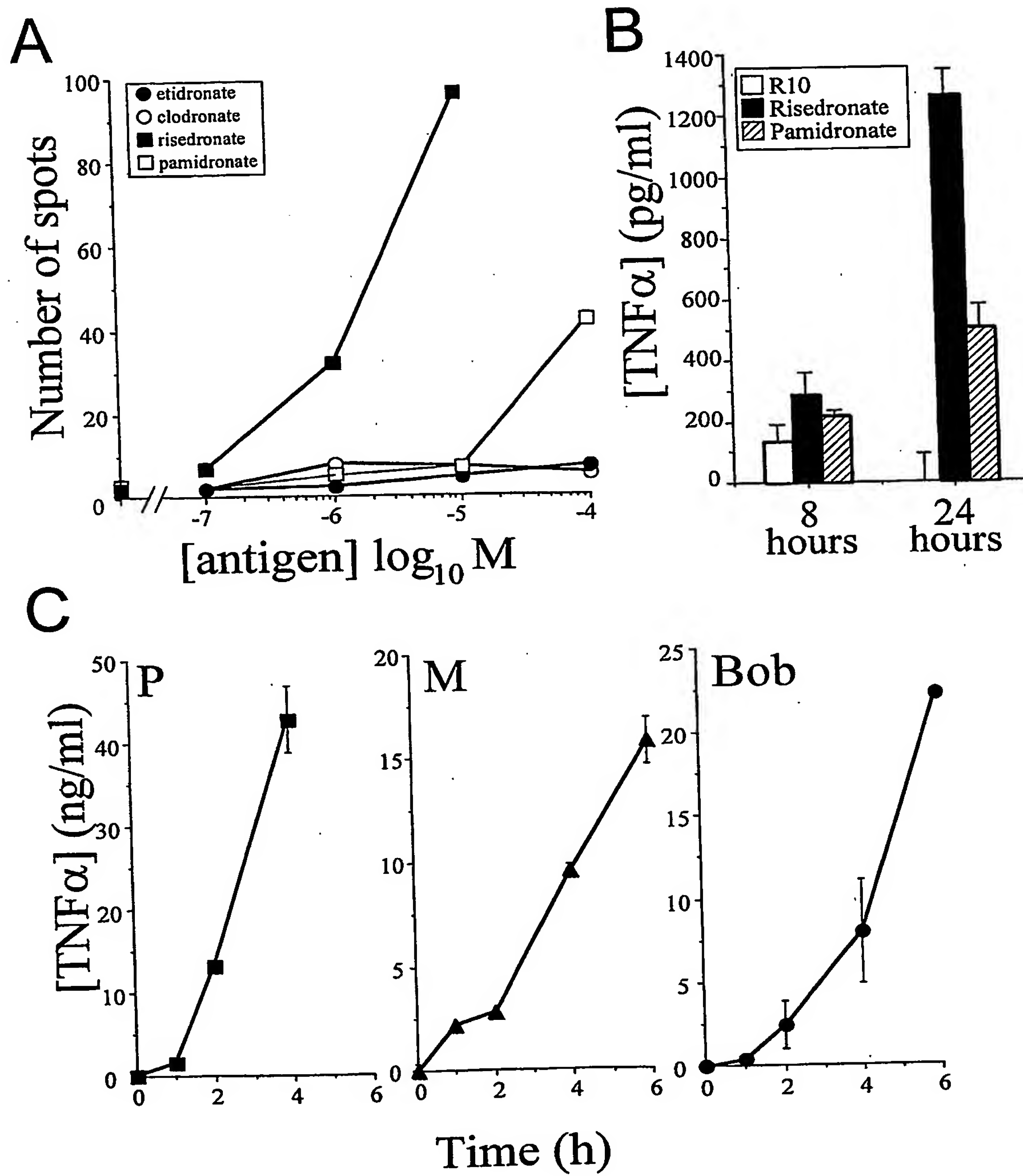


Figure 2

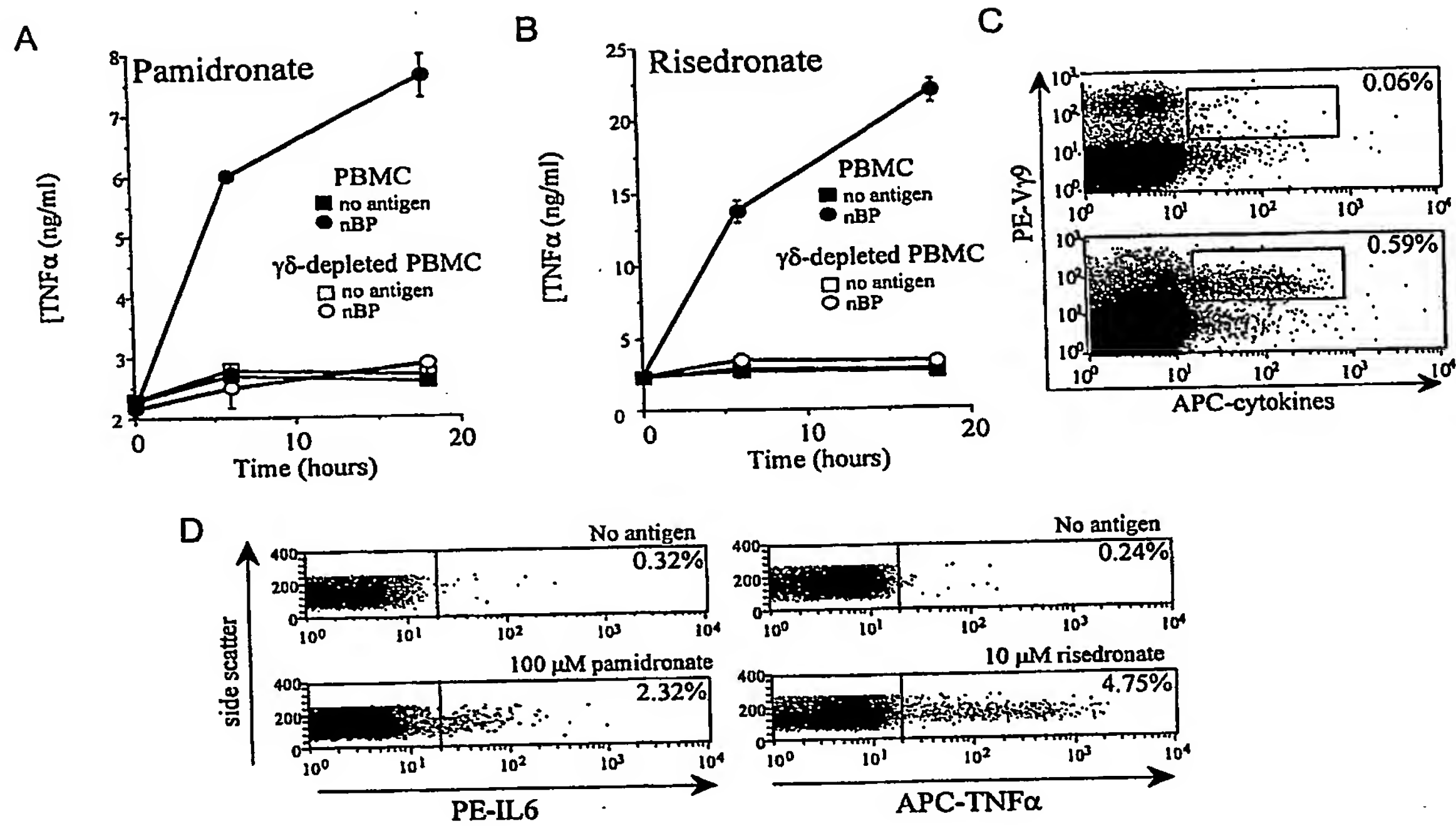
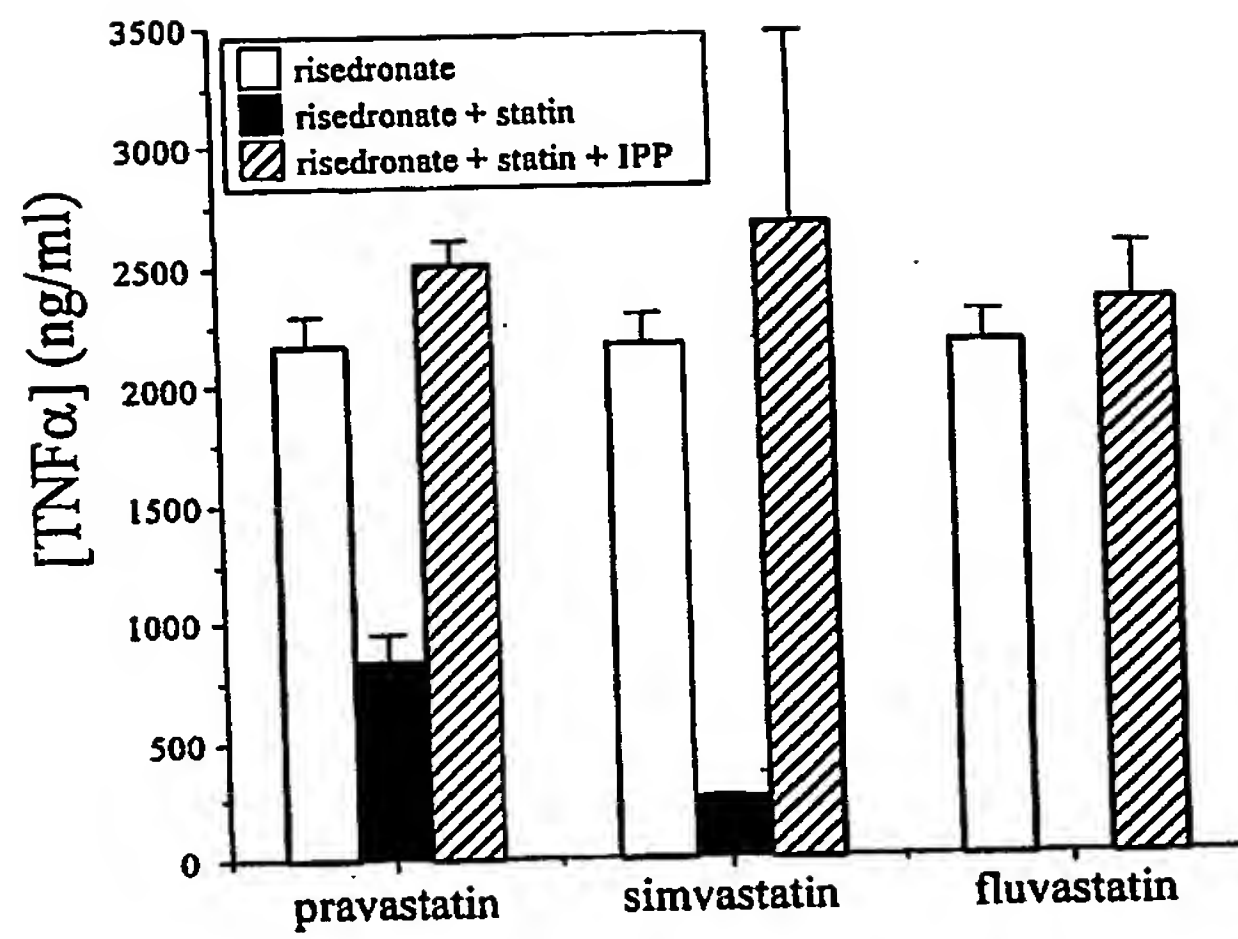
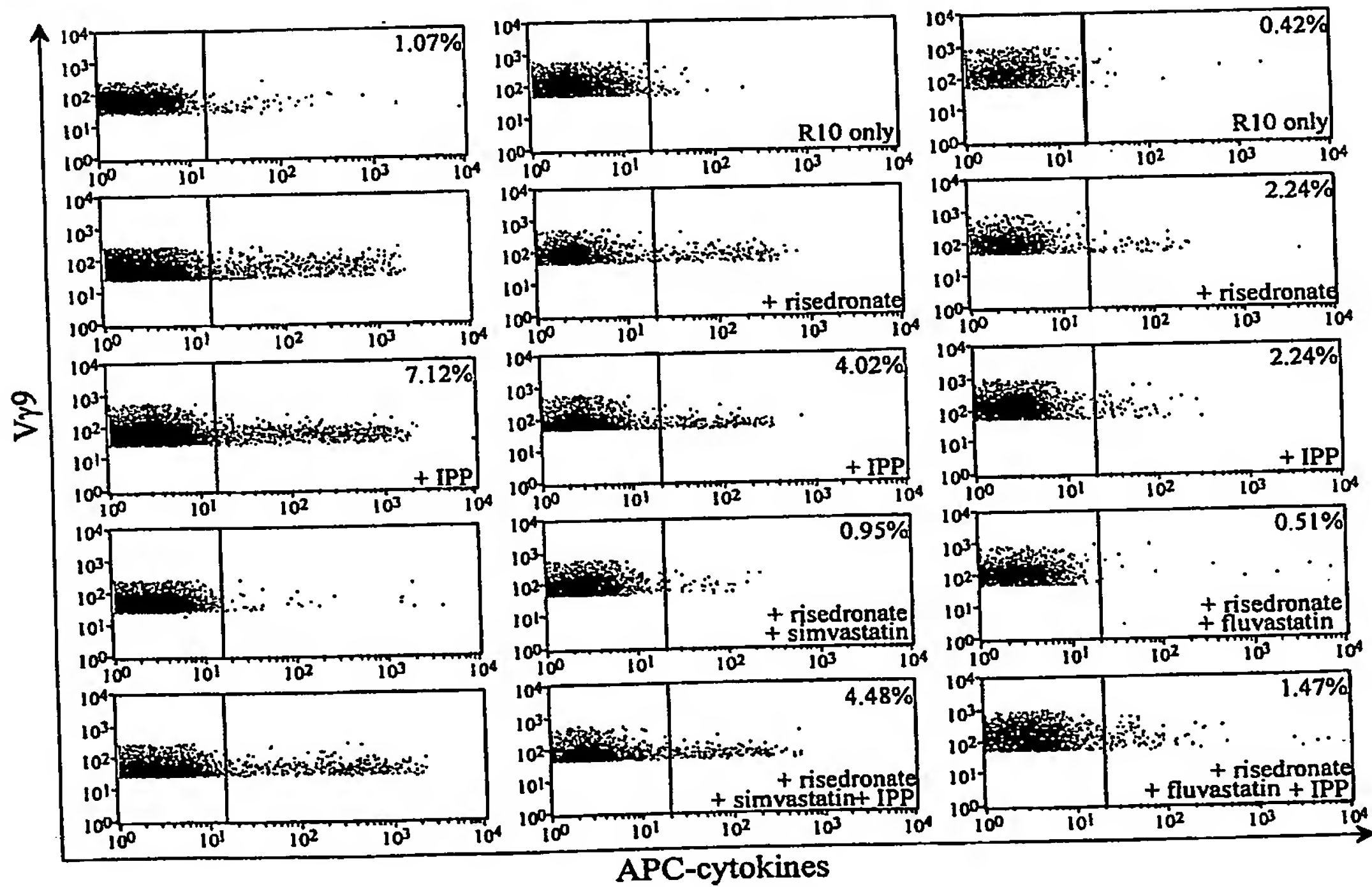


Figure 3

A



B



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